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CHARACTERIZATION OF WOUND-INDUCIBLE GENES ENCODING ENZYMES FOR TERPENOID BIOSYNTHESIS IN *MEDICAGO TRUNCATULA*

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Abstract:

In addition to having numerous applications and pharmaceuticals, terpenoids are an important class of defensive compounds that can accumulate in plants after pathogen infection or injury by insects. Sequences of DNA encoding putative terpene synthases and an oxidosqualene synthase, isolated from insect-damaged Medicago truncatula leaves, were selected from an expressed sequence tag (EST) data base. The cDNA clones were used as radio-labeled probes to analyze gene expression in leaves treated with factors known to trigger a defense response in plants. Transcript levels for all of the genes examined increased in response to artificial wounding, insect herbivory, and methyl jasmonate (meJA) treatments, whereas salicylic acid (SA) and glucose oxidase (GOA) had no measurable effects on transcript levels. Furthermore, the genome of M. truncatula was analyzed via DNA blots for an estimation of the -number of copies of enzyme isoforms and indicate that each of the enzymes examined is encoded by a single-copy gene or a small gene family. The results show that M. truncatula can serve as a valuable source for novel terpene synthase clones and potentially for strong wound-inducible regulatory elements.

Introduction:

Plants have unique responses to specific physical or chemical stimuli and this can be manifested by induction of genes putatively involved in a defense system. An example of a plant response to herbivory is demonstrated when lepidopteran larvae feed on a plant and volatile compounds are produced and systemically released from leaves. These volatile compounds can attract parasitoid wasps that are natural enemies of the herbivorous insect. In some cases wasps lay eggs in the lepidopteran larvae, and when they hatch, the wasp larvae devour the caterpillar from within (Turlings *et. al.*, 1995). Thus the plant is protected indirectly, through an induced mechanism, from further damage by the herbivore (Kessler and Baldwin, 2001).

One of the most abundant and common classes of induced volatile plant compounds released in response to insect herbivory is the terpenoids. For the induced production of volatile terpenoids, genes are possibly induced to express the proteins needed for volatile biosynthesis. The chemical pathways that lead to terpenoids begin with a five-carbon building block known as isopentenyl diphosphate (IPP). Two independent biosynthetic pathways can produce IPP, the mevalonate (MVA) pathway localized in the cytosol and the 2C-methyl erythritol 4-phosphate (MEP) pathway found in plastids. The five-carbon IPP units bond together in a head-to-head, head-to-tail, or head-to-middle fashion to form an acyclic prenyl phosphate. Specificity of the terpenoid produced is determined by the activity of the terpene synthase enzymes that convert the acyclic prenyl phosphate. Mono- and di-terpenes (C₁₀ and C₂₀ compounds, respectively) are thought to originate via the MEP pathway, whereas sesqui-, tri- (C₁₅ and C₃₀, respectively), and poly-terpenes are produced predominantly by the MVA pathway.

Terpenoids have a wide range of activities, and their applications range from flavorings and perfumes to pharmaceuticals. Individual sesquiterpenes have been shown to act as antimicrobial phytoalexins and as insect

antifeedants. One form of triterpenes, known as saponins, has antifeedant and antifungal properties that aid in plant defense (Haralampidis *et. al.*, 2001).

A plant may respond differently to mechanical damage than to actual insect damage. These differential responses can be due to insect-derived oral factors that are perceived by the plant. A protein component in the saliva of some insects, glucose oxidase (GOX), may trigger a response by the plant (Felton and Eichenseer, 1999). Plant defense responses can also be triggered by the perception of volatile compounds such as methyl jasmonate (meJA). Methyl jasmonate is produced constitutively in plants, but often increases in abundance as plants undergo stress. Plants release meJA, which serves as a signal to surrounding plants that an herbivore is feeding nearby. However salicylic acid (SA), a key modulator of systemic acquired resistance (SAR) to pathogens, can interfere with the meJA-centered defense pathway (Felton *et. al.*, 1999).

For insect herbivory experiments, *Spodoptera exigua*, the beet armyworm, was employed to feed on plants. This caterpillar can be a serious pest of many crops such as alfalfa, bean, broccoli, corn, cotton, soybean, and tomato to list a few. Gene isolation and expression studies used *Medicago truncatula*, an excellent model plant for genetic analyses. This self-fertilizing legume possesses a small diploid genome and is easy to transform. It also has a relatively short generation time, allowing for more rapid genetic studies. In addition, a large-scale genomics project in *M. truncatula* is underway, including the sequencing of expressed mRNAs. To date, over 140,000 expressed sequence tags (ESTs) are available in a public database (<http://www.tigr.org/tdb/mtge/>); this includes nearly 10,000 ESTs that were derived from *S. exigua*-injured *M. truncatula*.

To understand plant defense responses to insects, it is important to characterize the regulation of genes that encode the enzymes that produce terpenes and saponins. Based on sequence similarities with known genes from other plant species, we selected three putative terpene synthase cDNA clones and one putative β -amyrin synthase (responsible for saponin biosynthesis) clone from the EST database. Accumulation of mRNA for each gene was measured in response to several types of wounding and treatment with meJA, SA, and GOX.

Materials and Methods:

Plant and insect maintenance

M. truncatula, line A 17, was grown under standard conditions in a growth chamber at 24°C with a 16:8 hour light: dark regime. Fertilizer was administered at 2-week intervals. All treatments were carried out in a greenhouse and were started at 0900 hours. Eggs of *S. exigua* were obtained from the USDA Gast Rearing Lab (Starkville, Miss.). Larvae were maintained on an artificial diet under conditions at approximately 22°C.

DNA probes

DNA probes were derived from a cDNA library of *M. truncatula* leaves that had been subjected to *S. exigua* herbivory. The clones were identified in a search of the *M. truncatula* EST database based on sequence similarity with characterized terpene and beta-amyrin synthases from other plant species. Four clones were chosen for analysis. The terpene synthase clones and their Genbank accession numbers, were: A4 (accession no. BF639687); A7 (accession no. BF640252); and A10 (accession no. BE321953). The putative β -amyrin synthase clone was designated B3 (accession no. BF642680).

Insect treatment

Insects were placed on plants and allowed to feed for 24 hours before samples were taken. Leaves that had been damaged by the insect were "local," and undamaged leaves on the same trifoliate as an insect-damaged leaf

were "systemic." Artificial wounding was conducted by cutting leaves with scissors, and only the locally damaged leaves for this type of treatment were collected for sampling after 6 hours. Control samples came from leaves of undamaged plants.

Chemical treatments

For all meJA treatments, intact plants were placed in 18 L glass chambers. Cotton swabs with volumes of 0.5 μ L, 1.0 μ L, and 2.0 μ L meJA (calculated volatile concentration 0.125 μ M, 0.25 μ M, and 0.5 μ M, respectively) were inserted into the soil next to each plant in separate chambers and the open-end bottom of each chamber was covered with a layer of foil and cheesecloth. A control plant was placed in a glass chamber with no added meJA. The plant was removed from the chamber after 1 hour and leaf samples were taken after 6 hours. A second experiment was conducted using the same technique but over an 18-hour time period.

Glucose oxidase was applied to leaves after wounding with a tracing wheel. As the wheel was rolled across the leaves, it punctured small holes in the plant, and 20 μ L of 1.3 mg/mL GOX solution was pipetted onto the leaves. This level of GOX approximates the concentration measured in *Spodoptera* labial gland extracts (H. Eichenseer, personal communication). Local and systemic tissues were collected at 1 and 6 hours after the treatment. For testing the effects of SA, solutions of SA in water were sprayed on plants at 2 mM, 4 mM, and 8 mM. For control treatments, the solvent with no SA was used. Samples were taken at 1 and 6 hours after treatment.

Nucleic acid analysis

Leaves were collected, immediately chilled in liquid nitrogen, and stored at -70°C until analysis. Total RNA was extracted using TriReagent (MRC, Inc. Cincinnati, Ohio) and separated on 1% agarose formaldehyde gels. The RNA was transferred to nylon membranes and hybridized with radio-labeled probes (Church and Gilbert, 1984). Insert DNA from individual cDNA clones was amplified via polymerase chain reaction and radio-labeled with 32 P in random-primer reactions (Sambrook *et al.*, 1989).

M. truncatula genomic DNA was isolated according to Junghans and Metzlaff (1990). DNA was digested in individual reactions with *Bam*HI, *Eco*RI, or *Hind*III overnight at 37°C. Cleaved genomic DNA was separated on a 1.0% agarose gel, denatured, and transferred to a nylon membrane (Sambrook *et al.*, 1989). Hybridizations were carried out as for RNA blots.

Results and Discussion:

EST clone selection

A search of the *M. truncatula* EST database revealed the presence of at least three putative terpene synthase clones and one β -amyrin synthase clone that were derived from insect-damaged leaves. Functional assignment of the clones was based on the presence of highly conserved sequence domains for each type of enzyme. Terpene synthase clones were designated A4, A7, and A 10. The A4 sequence is predicted to encode a plastid transit signal at its amino terminus, indicating that it probably encodes a mono- or di-terpene synthase. The A7 and A 10 clones bear highest sequence similarity to known sesquiterpene synthase clones, whereas the B3 clone is highly similar to a characterized oxidosqualene synthase, namely β -amyrin synthase. Consistent with their putative enzymatic functions, none of the A7, A 10, or B 3 sequences contains a predicted plastid transit sequence. Full-length cDNA clones for each sequence were obtained and utilized to characterize transcript accumulation and gene copy number.

Wounding induces transcript accumulation

Measurement of RNA accumulation demonstrates that genes encoding terpene synthases and β -amyrin synthase were induced by artificial- and insect-wounding. For most of the genes examined, the highest levels of transcripts are observed in leaves injured by insect herbivory (Fig. 1). For each gene, very low levels of RNA were present in undamaged leaves. Artificial damage also caused an increase in transcript accumulation, but generally not to the same degree as insect damage. This was not the case for A10, where the highest transcript levels were observed after artificial damage. For clones A7 and AIO, two bands were consistently observed on RNA blots. This suggests there might be multiple forms of similar transcripts, derived from independent genes that are cross-hybridizing on the membranes. For the AIO transcripts, the two bands were always observed at similar levels, suggesting that if they are derived from independent genes, these genes must be coordinately regulated. Transcripts for each gene were also induced in systemic tissues of insect-damaged plants, although not to the same level as in damaged leaves. The A7 transcript was consistently the most strongly induced of all the genes examined. Insect herbivory is known to often elicit a greater plant response than artificial damage, probably due to the differing types of wounding or the presence of elicitor compounds associated with the insect (Korth and Dixon, 1999 Walker-Simmons, *et. al.*, 1984). The enzyme products of the genes examined here are predicted to be involved in the biosynthesis of defense compounds, so it is not surprising that transcripts accumulated to high levels following wounding. Probing for the constitutively present ribosomal RNA indicated that equivalent amounts of total RNA were present in each gel lane.

Gene induction by methyl jasmonate

Treatment with meJA also led to transcript accumulation for each of the genes examined. Transcript levels were low in untreated samples, but RNA accumulation increased dramatically when plants are exposed to the lowest concentration of volatile meJA applied, 0.125 μ M (Fig. 2). Levels of A4 transcripts increased with increasing levels of meJA, whereas transcripts for the other genes were somewhat lower with increasing levels of meJA. Temporal expression of transcript accumulation was tested for the terpene synthase clones after exposure to volatile 0.25 μ M meJA (Fig. 3). The A4 transcripts were present to some degree even in untreated control samples in this experiment, but the RNA blot seemed to indicate that transcripts accumulated to higher levels between 2-6 hours after initial exposure to meJA. For A7 and AIO, transcript levels clearly increased with time, and returned to normal levels by 18 hours after the initial exposure. As in wound experiments, the A7 transcripts were the most abundant, and the A7 transcripts also were induced earlier than the other genes tested.

Glucose oxidase and salicylic acid treatments

Glucose oxidase is the most abundant protein found in labial gland saliva of lepidopteran insect larvae (Eichenseer and Felton, 1999). This enzyme has been shown to affect plant responses to chewing caterpillars and to wounding when it is applied to a wound site (J. Bede and G. Felton, personal communications). Addition of GOX did not have any effect on transcript accumulation for any of the genes used in this study; levels of RNA in leaves treated with GOX did not differ significantly from those treated with water (data not shown).

In addition, treatment with SA ranging from 2-8 mM did not affect transcript accumulation (data not shown), therefore SA alone seems not to be directly involved in the regulation of these genes.

Enzymes encoded by small gene families

Probing *M. truncatula* genomic DNA with the selected cDNAs revealed that this species contains low copy numbers of the genes examined. Banding patterns indicate that the terpene synthase sequences are present in one to three copies each, whereas sequences hybridizing to the 133 β -amyrin synthase clone are present in three

to four copies (Fig. 4). Although the genes examined here all encode well-conserved protein domains, it is well established that enzymes similar in primary sequence can differ greatly in terms of the specific products that they synthesize (Bohlmann *et. al.*, 1998). Therefore, independent genes that cross-hybridize on DNA blots might encode enzymes with very different specificities. At the very least, our data indicate that there is a low degree of genetic redundancy for the sequences that we tested via genomic DNA blots. Knowledge of the copy number of these genes will be important if efforts are made to isolate genomic promoter sequences.

Conclusions:

The results from our experiments reveal that insect herbivory and chemical treatments can cause systemic gene responses. Systemic leaves of wounded plants accumulated transcripts for terpene and β -amyrin synthases, showing that gene-induction signals are being transported through the plant. In wounded leaves, the highest levels of transcript accumulation were generally observed after insect herbivory. This result is indicative of the presence of specific insect-derived elicitors or a unique type of damage during chewing by lepidopteran larvae as compared to mechanical wounding.

Treatments with meJA, a central modulator of wound responses in most plant species, showed that this plant hormone can regulate expression of defense genes in *M. truncatula*. Addition of volatile meJA to intact plants caused a rapid and transient accumulation of terpene synthase- and β -amyrin synthase-encoding genes.

The addition of GOX and SA, compounds known to affect expression of some plant defense genes, did not affect accumulation of any of the genes examined in this study. Although GOX can repress levels of some plant-defense gene transcripts (J. Bede and K. Korth, unpublished data), we did not see any differences in transcript levels when comparing wounded leaves with and without added GOX.

Understanding the regulation of the genes described here might aid ultimately in manipulation of plant defense responses or in the biosynthesis of valuable terpenoid compounds. The role of these genes' products in defense is suggested by the strong and rapid induction of transcripts that occurred following insect herbivory. With the basic characterization reported here, targeted studies of the function of these genes in defense can be carried out. The enzymes that these genes encode, or the promoter sequences that control their regulation, might provide valuable tools in production of plants that are more insect-resistant. This work clearly demonstrates that *M. truncatula* can serve as a new source of novel and valuable genes encoding enzymes involved in plant defense and terpenoid biosynthesis.

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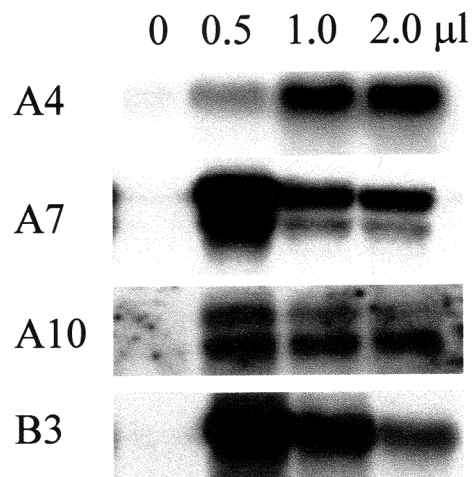


Figure 1. Transcript accumulation as indicated by RNA blots, in leaves following artificial damage with scissors ("wound"), or *S. exigua* herbivory. Leaves were collected at 6 hours after the initial damage. Membranes were hybridized with the indicated probes, and bands were visualized via autoradiography.

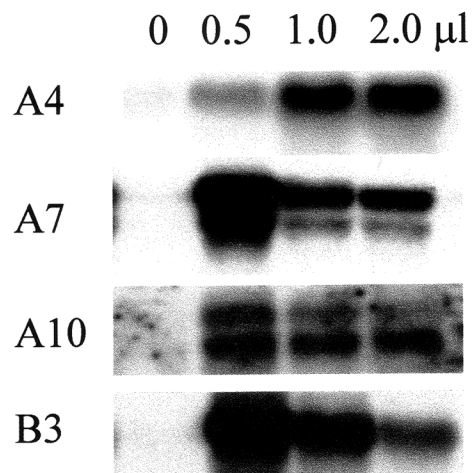


Figure 2. Transcript accumulation in leaves following exposure to differing levels of meJA. Leaves were collected 6 hours after intact plants were placed in a glass chamber with 0, 0.5 μL (0.125 μM), 1.0 μL (0.25 μM), or 2.0 μL (0.5 μM) meJA for 1 hour. Membranes were hybridized with the indicated probes, and bands were visualized via autoradiography.

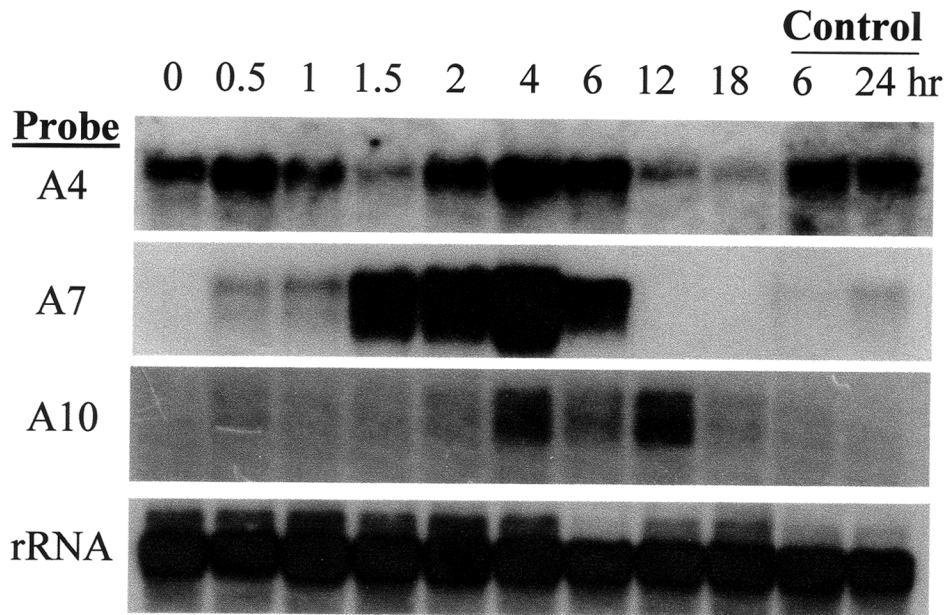


Figure 3. Transcript accumulation in leaves following exposure to 1.0 μ L (0.25 μ M) of meJA. Leaves were collected at the time indicated, after intact plants were placed in a glass chamber for 1 hour with meJA. Membranes were hybridized with the indicated probes, and bands were visualized via autoradiography.

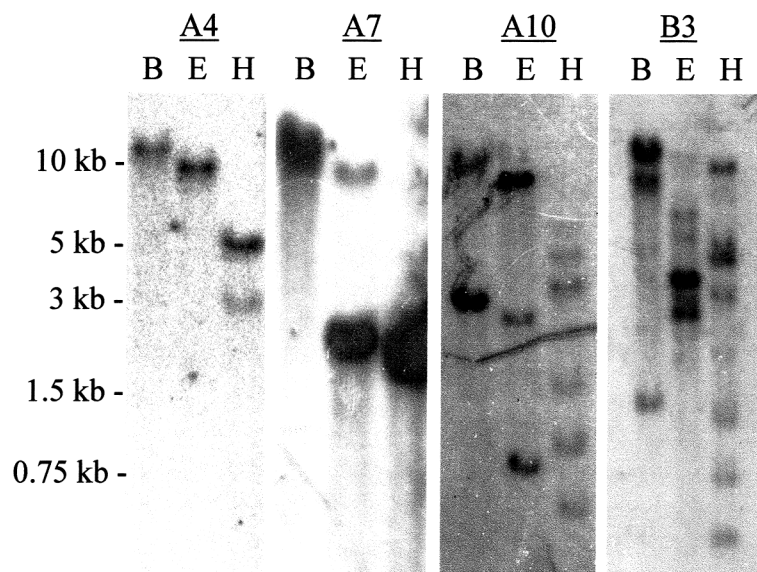


Figure 4. DNA blot analysis of terpene synthase and β -amyrin synthase clones. Genomic DNA from *M. truncatula* was digested with *Bam*HI (B), *Eco*RI (E), or *Hind*III (H) and separated on a 1% agarose gel. Positions of DNA size markers are indicated at left. Identical membranes were hybridized with the indicated probes, and bands were visualized via autoradiography.